

**THE EFFECTS OF CURCUMIN ON THE EXPRESSION OF P53 AND BCL-2
ONCOPROTEINS ON THE HUMAN BREAST CARCINOMA CELL LINE
MDA-MB 468**

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MDA-MB 468

A THESIS SUBMITTED TO
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BY
ANGELIN S. SHAJAN
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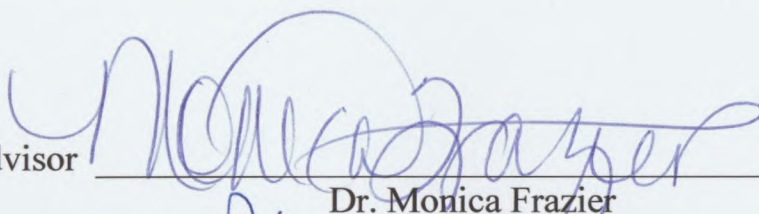
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The Effects of Curcumin on the Expression of p53 and Bcl-2 Oncoproteins on the
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by
Angelin S. Shajan

A Thesis Submitted in Partial Fulfillment of
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for Honors in the degree of
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in
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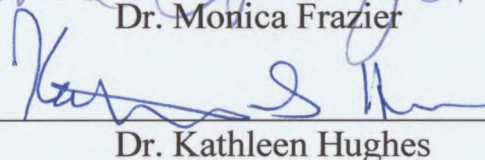
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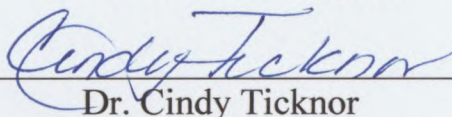
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ABSTRACT

Advisors Dr. Monica Frazier and Dr. Kathleen Hughes

We studied the effects of curcumin on the proliferation and viability of MDA-MB-468 breast cancer cells and its effects on the expression of Bcl-2 and p53 proteins in these cells. Based on previous studies, we predicted a dose-dependent increase in p53 (tumor suppressor) expression, and a decrease in Bcl-2 (anti-apoptotic oncoprotein) expression. Briefly, MDA-MB-468 cells were seeded into 12-well plates at 2.0×10^4 cells/well and allowed to grow for 72 hrs. Cells were treated with curcumin at 0, 25, 50 and 100 μ M in triplicate at time zero and cultured for another 24 hours. All data were obtained using a Muse Cell Analyzer. Proliferation and viability data were determined using an Annexin V and Dead Cell Kit, p53 expression was determined using a Multi-Colored DNA Damage Kit, and Bcl-2 expression was determined using a Bcl-2 Activation Dual Detection Kit. Results show a significant dose-dependent increase in both cell death and proliferation. However, there was no significant difference in expressions of both p53 and Bcl-2 oncoproteins across the four treatments. Future studies will investigate additional mechanisms by which curcumin induces these apoptotic effects.

INDEX WORDS: Apoptosis, bcl-2 protein, curcumin, p53 protein.

TO MY BIGGEST SUPPORTERS

Dear mom and dad: Words cannot begin to describe my appreciation and gratitude for all you have done for me. Your love and support has helped me not only get through college, but also through life. While I honestly do not think I will ever comprehend the true depth of your sacrifice, I can promise that I will continuously try. I will always strive to make you proud because you truly deserve the best. I love you both so much. Love, Angelin.

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1.1.1. Project Overview

Project Overview: This project aims to develop a comprehensive system for managing and analyzing data from various sources. The system will be designed to handle large volumes of data, provide real-time analytics, and support decision-making processes. The project is divided into several phases, including requirements gathering, system design, development, testing, and deployment. The final goal is to create a robust and scalable solution that meets the needs of the organization.

Chapter I
Introduction

Chapter I: Introduction. This chapter provides an overview of the project and its objectives. It discusses the importance of the project and the challenges it presents. The chapter also introduces the key concepts and terminology used throughout the document. The introduction is divided into two main sections: a general overview and a detailed description of the project's scope and goals. The general overview provides a high-level summary of the project, while the detailed description provides more specific information about the project's objectives, scope, and deliverables. The introduction concludes with a brief summary of the chapter's content and a preview of the next chapter.

1.1.2. Overview

Overview: This section provides a high-level overview of the project and its objectives. It discusses the importance of the project and the challenges it presents. The overview is divided into two main sections: a general overview and a detailed description of the project's scope and goals. The general overview provides a high-level summary of the project, while the detailed description provides more specific information about the project's objectives, scope, and deliverables. The overview concludes with a brief summary of the chapter's content and a preview of the next chapter.

1.1.1 Breast Cancer

Recent statistics show that breast cancer is the most common and the second leading cause of malignancy-related deaths among women worldwide (Crawford and Richard, 2013; Jemal et al., 2011). In the United States alone, more than 200,000 new cases are diagnosed annually and approximately 40,000 deaths occur as a result of this malignancy (Crawford and Richard, 2013; Jemal et al., 2011).

Cancer occurs when cells begin to proliferate uncontrollably due to genetic mutations and/or abnormal proliferation signals (Watts, 2013). During puberty, the hormone estrogen signals the development of breast tissue and the hormone progesterone stimulates the growth of lobular tissue (BreastCancer.org, Watts, 2013). When abnormal cell growth arises in ductal breast tissue, it is termed ductal carcinoma in situ, DCIS and when abnormalities occur in lobular tissue, it is termed lobular carcinoma in situ, LCIS (BreastCancer.org, Watts, 2013). Breast cancer onset can take a few years, beginning with a cluster of cells that are unable to follow the “rules” of the cell cycle progression, to a later accumulation of increased mutations that make these cells capable of unrestricted proliferation (BreastCancer.org).

1.1.2 Curcumin

Curcumin, the active ingredient of turmeric (curry spice), is believed to be associated with reduced incidence rates of breast cancer among women in Southeast Asian countries (Nagaraju et al. 2012). Curcumin (diferuloylmethane) is a yellow-colored polyphenol that is extracted from the *Curcuma longa* plant, commonly known as turmeric (Liu and Chen, 2013; Welkin et al., 2011). It has been consumed as a dietary spice for centuries and has played a key role in Ayurvedic medicine. Curcumin is known to possess a variety of beneficial and

therapeutic properties such as anti-oxidant, anti-inflammatory, anti-metastatic and anti-proliferative activities (Liu and Chen, 2013; Wilken et al., 2011). Recent studies have also proven curcumin's anti-cancer properties via its suppression of gene products such as c-myc, Bcl-2, and interleukins, its target of biological pathways, inhibition of growth factors, transcription factors and cell adhesion molecules involved in mutagenesis, cell cycle regulation, gene expression, apoptosis, metastasis, and tumorigenesis (Wilken et al., 2011, Liu and Chen 2013; Cine et al., 2013).

1.1.3 p53 protein and cancer

The tumor suppressor gene p53, often referred to as the “guardian of the genome”, is required for checkpoint control during cell cycle progression (Meikrantz and Schlegel, 1995). During the cell cycle, DNA damage is detected at two checkpoints: the G₁-S phase and the G₂-M phase (Merritt et al., 1997). Depending on the type and extent of DNA damage, p53 can initiate one of three pathways: cell cycle arrest at a checkpoint, repair of the damaged cell, or apoptosis of the cell (Elmore, 2007; Figure 1). However, in cancer cells, these pathways are defective and therefore the cells lack the ability to detect damage and thereby initiate an appropriate response as needed. This can eventually lead to genetic instability and uncontrolled cell growth resulting in tumors (Elmore, 2007). When DNA damage occurs, the kinase ATM is the first protein to be phosphorylated. Once phosphorylated, ATM can activate various downstream gene products, including p53, SMC1, NBS1, and Histone H2A.X (Burma et al., 2001; Figure 1). One of these factors, the H2A.X, is a key indicator of the levels of DNA damage, specifically double-stranded DNA breaks. As the level of DNA damage rises, the level of phosphorylated H2A.X also rises and begins to aggregate at locations where damage is detected (Burma et al., 2001). H2A.X can

then activate various downstream factors such as p53 to initiate repair of the damaged cell. The major role of the checkpoint controlling entry into S phase of the cell cycle is to prevent damaged cells from replicating damaged DNA. This is controlled via the p53 protein, which induces either cell cycle arrest in G₁ or cause apoptosis (Merritt et al., 1997; Figure 1). Although the p53-mediated G₁ arrest has been commonly studied, p53 regulation at the G₂ phase has also been found to occur (Guillouf et al., 1995).

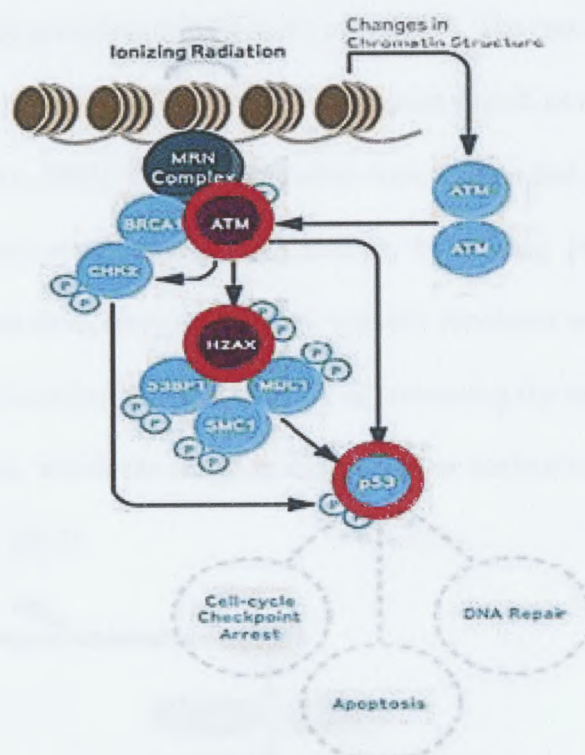


Figure 1. A proposed pathway by which ATM and H2A.X activate p53 to induce DNA repair, cell cycle arrest, and apoptosis¹⁷.

1.1.4 Bcl-2 protein and cancer

Bcl-2 is a member of the much larger Bcl-2 family that controls apoptosis by regulating the proteins responsible for mitochondrial membrane permeability (Jin and El-Deiry, 2005). The Bcl-2 family consists of two protein sub-groups, the pro-apoptotic proteins and the anti-apoptotic

proteins. Bcl-2 is an anti-apoptotic family member that acts by forming a complex with pro-apoptotic proteins such as Bax, thereby sequestering them and inhibiting their insertion into the mitochondrial membrane (Adams and Cory, 2007). When an apoptotic signal is received, Bcl-2 is supposed to release Bax, allowing Bax to insert into the mitochondria and release cytochrome C into the cytoplasm, promoting caspase activation and cell death (Figure 2). However, in cancer cells, apoptosis is often prevented due to an up-regulation of anti-apoptotic Bcl-2 proteins or dysfunction of pro-apoptotic proteins (Adams and Cory, 2007). The ratio of anti-apoptotic Bcl-2 proteins is often very high in various hematologic malignancies such as non-Hodgkin's lymphoma (Jin and El-Deiry, 2005). Preclinical studies have shown that Bcl-2 serves a major role in regulating the intrinsic apoptotic signaling pathway by binding pro-apoptotic molecules and thereby preventing them from carrying out their specific functions within the mitochondria (Jin and El-Deiry, 2005). Therefore, Bcl-2 is capable of promoting the survival of regulatory B-cells by inhibiting apoptosis, which can result in chemotherapy resistance in various hematologic cancers (Adams and Cory, 2007).

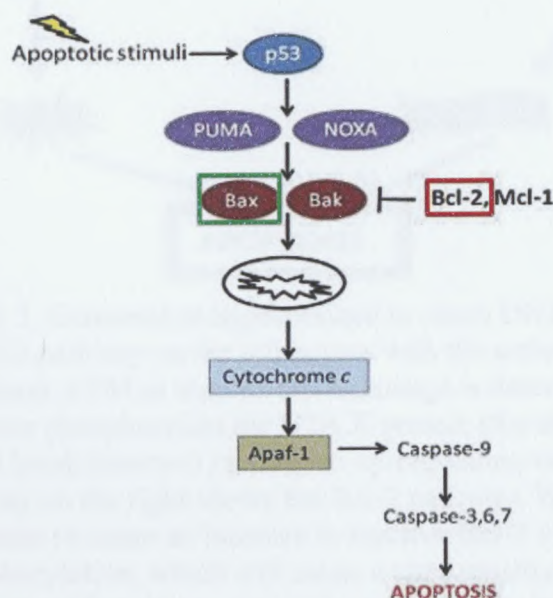


Figure 2. A proposed pathway by which Bcl-2 and Bax interact to induce apoptosis after cell damage is detected²⁰.

1.1.5 Aims of our Study

The goals of this study were to determine the anti-proliferative and apoptotic effects of curcumin on MDA-MB 468 cells and to determine how curcumin affects the abundance of two specific apoptotic and cell cycle proteins: p53 and Bcl-2. **We hypothesized a dose-dependent increase in cell death and a dose-dependent decrease in cell proliferation following curcumin treatment. We also hypothesized that curcumin would cause an up-regulation of p53 expression and a down-regulation of Bcl-2 activation (Figure 3).**

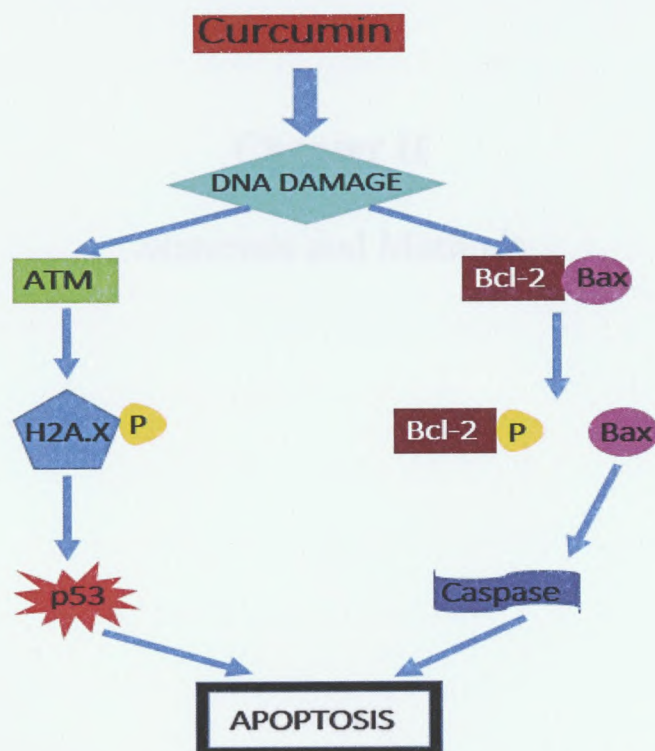


Figure 3. Curcumin is hypothesized to cause DNA damage. The p53 pathway on the left begins with the activation of the kinase ATM as soon as DNA damage is detected. ATM will then phosphorylate the H2A.X protein (the double-strand break detector) causing an up-regulation of p53. The pathway on the right shows the Bcl-2 pathway. We predict curcumin to cause an increase in inactive Bcl-2 via phosphorylation, which will cause a conformational change and prevent Bax (a pro-apoptotic protein) to be bound to Bcl-2. This will allow Bax to form its own complex and activate caspases that will eventually lead to apoptosis.

Chapter II

Materials and Methods

Materials

MDA-MB-468 breast adenocarcinoma cells were obtained from the American Type Cell Culture Collection (ATCC, Manassas, VA) and cultured in Leibovitz L-15 Media (Hyclone, Logan, UT) supplemented with 10% Fetal Bovine Sera (Atlanta Biologicals, Atlanta, GA) and 1% Antibiotic/antimycotic (Invitrogen, Grand Island, NY). MDA-MB-468 were grown at 37°C in a free gas exchange with atmospheric air. Curcumin (diferuloylmethane) and dimethyl sulfoxide (DMSO) were obtained from GE Healthcare Bio-Sciences (Pittsburgh, Pennsylvania). Annexin V and Dead Cell kit, Multi-Colored DNA Damage kit, and Bcl-2 Dual Detection kit were obtained from EMD Millipore Muse® (Billerica, Massachusetts).

Cell Treatment

Curcumin was first dissolved in DMSO (Sigma-Aldrich, St. Louis, MS) as a 10 mM stock solution and subsequently diluted in L-15 culture medium. Medium containing the same amount of DMSO was used as control at concentrations not exceeding the 0.1% v/v of the culture medium. Cells were incubated with curcumin at 0, 25, 50, and 100 μ M, at three time points namely 24, 48, and 72 h. Briefly, MDA-MB-468 cells were inoculated at a density of 2×10^4 cells/well in a 12-wellplate for 72 h in 1 mL of L-15 medium. The culture supernatant was then removed and medium containing the above mentioned curcumin concentrations were added to cells and subsequently incubated for 24 h.

CONTROL	25 μ M	50 μ M	100 μ M
L-15 Media	Tube C	Tube B	Tube A
L-15 Media	Tube C	Tube B	Tube A
L-15 Media	Tube C	Tube B	Tube A

Figure 4. Cell treatment in 12-well plate.

Annexin V and Dead Cell Kit

This kit was used to determine percentage of cell death and proliferation. Cells were detached from the plate using a cell scraper and 100 μ L of the cell suspension was placed into microcentrifuge tubes. Next, 100 μ L of Muse Annexin V and Dead Cell Reagent was added into each tube. Samples were mixed thoroughly using a vortex at medium speed for about 3 to 5 seconds. Finally, samples were stained for 20 min. at RT and analyzed using a Muse Cell Analyzer.

Multi-Color DNA Damage Kit

This kit was used to indirectly measure p53 expression. Briefly, cells were centrifuged at 300 x g for 5 min. and washed once with 1X PBS. Following centrifugation, cells were fixed with Fixation Buffer and permeabilized with Permeabilization Buffer for 10 min. on ice and washed with PBS as before. Cells were then treated with 10 μ L of the Anti-phospho-ATM /Anti-

phospho-Histone H2A.X antibody cocktail. Samples were incubated in the dark for 30 min. at room temperature. Following incubation, cells were centrifuged at 300 x g for 5 min. and washed with 1X Assay Buffer. Cells were then resuspended in 200 μ L 1X Assay Buffer and analyzed on a Muse Cell Analyzer.

Bcl-2 Dual Detection Kit

This kit was used to measure percentage of Bcl-2 protein activation. The procedure for this assay was exactly as the Multi-Color DNA Damage Kit, except cells were fixed and permeabilized for 5 min. and an antibody cocktail consisting of a phospho-specific anti-phospho-Bcl-2 +anti-Bcl-2 was used.

Statistical Analyses

The independent variable in the experiment was the curcumin concentration. The dependent variables were percentage of cell death, cell proliferation, DNA Double-strand breaks, and Bcl-2 inactivation. Data were expressed as means \pm 1 SD. Statistical significance of differences between means was determined by 1-way ANOVA and a Least Significant Differences (LSD) Post-Hoc test. Probability values of $p < 0.05$ were considered statistically significant.



Chapter III

Results and Discussion

Figure 5. Schematic diagram of a single-stage, single-pass, cross-flow, counter-current, liquid-liquid extraction process. The diagram shows a rectangular extraction column with two horizontal mixing stages. The top stage is labeled 'Feed' and the bottom stage is labeled 'Extract'. Arrows indicate the flow of the feed liquid from left to right and the extract liquid from right to left. A central vertical line represents the interface between the two liquid phases.

Figure 6. Schematic diagram of a single-stage, single-pass, cross-flow, counter-current, liquid-liquid extraction process. The diagram shows a rectangular extraction column with two horizontal mixing stages. The top stage is labeled 'Feed' and the bottom stage is labeled 'Extract'. Arrows indicate the flow of the feed liquid from left to right and the extract liquid from right to left. A central vertical line represents the interface between the two liquid phases.

Results

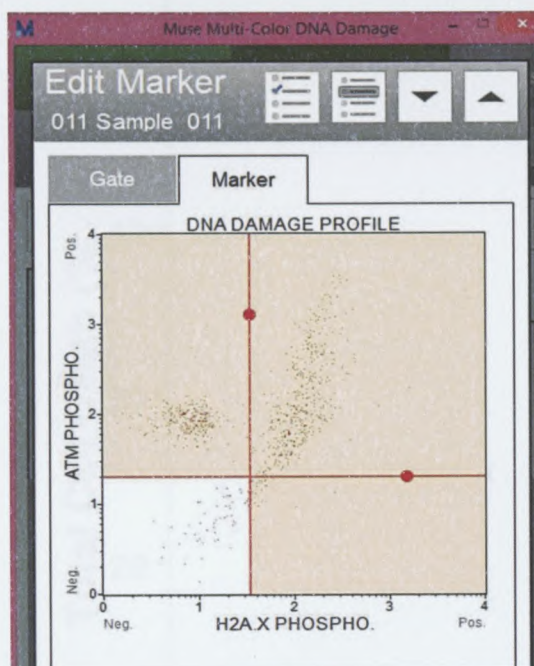


Figure 5. Results of a sample acquired using Muse® Cell Analyzer for the Multi-Color DNA Damage assay. The lower left quadrant shows percentage of negative cells that had no DNA damage. The lower right quadrant shows percentage of H2A.X- activated cells. The upper left shows percentage of ATM-activated cells, and the upper right indicates percentage of DNA double-strand breaks via dual activation of both ATM and H2A.X. This is the quadrant we were interested in since p53 expression involves the phosphorylation of both ATM and H2A.X.

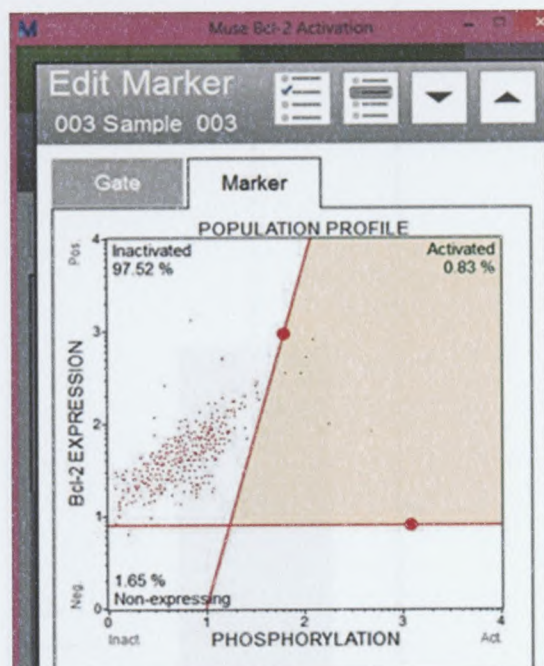


Figure 6. Results of a sample acquired using Muse® Cell Analyzer for the Bcl-2 Activation Assay. The lower left quadrant shows percentage of non-expressing cells. The upper right quadrant indicates percentage of activated cells via phosphorylation, and the upper left shows percentage of inactivated cells. This is the quadrant we were interested in since inactivation of Bcl-2 allows Bax to form a complex where it can induce apoptosis in the cells via activation of a series of downstream proteins.

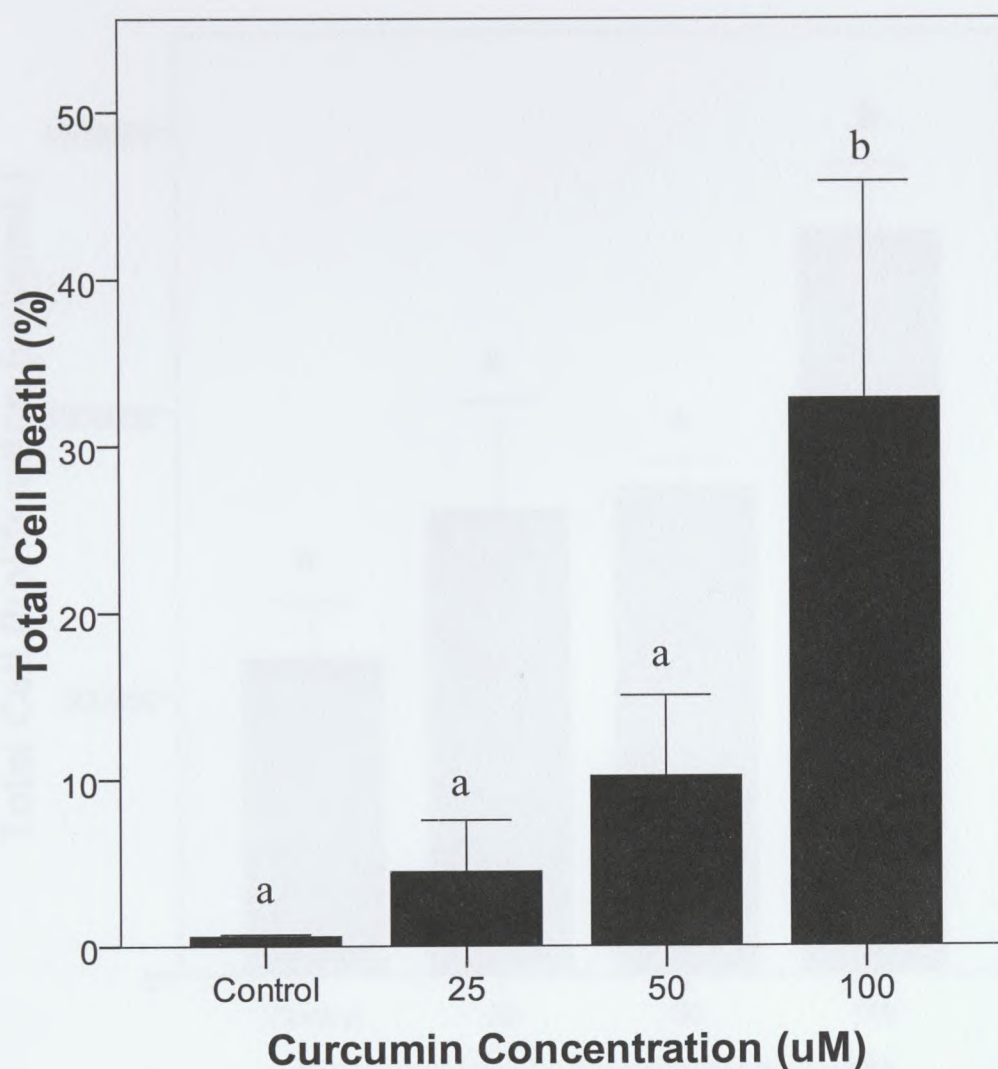


Figure 7. Effect of curcumin on average (± 1 S.E) death of MDA-MB 468 cells. The x-axis corresponds to the different concentrations of curcumin measured in μM (0, 25, 50, and 100) and the y-axis corresponds to the total cell death percentage. Cells were treated with the above curcumin concentrations for 24 h. Cell death was determined using the Annexin V Dead Cell kit and a Muse Cell Analyzer.

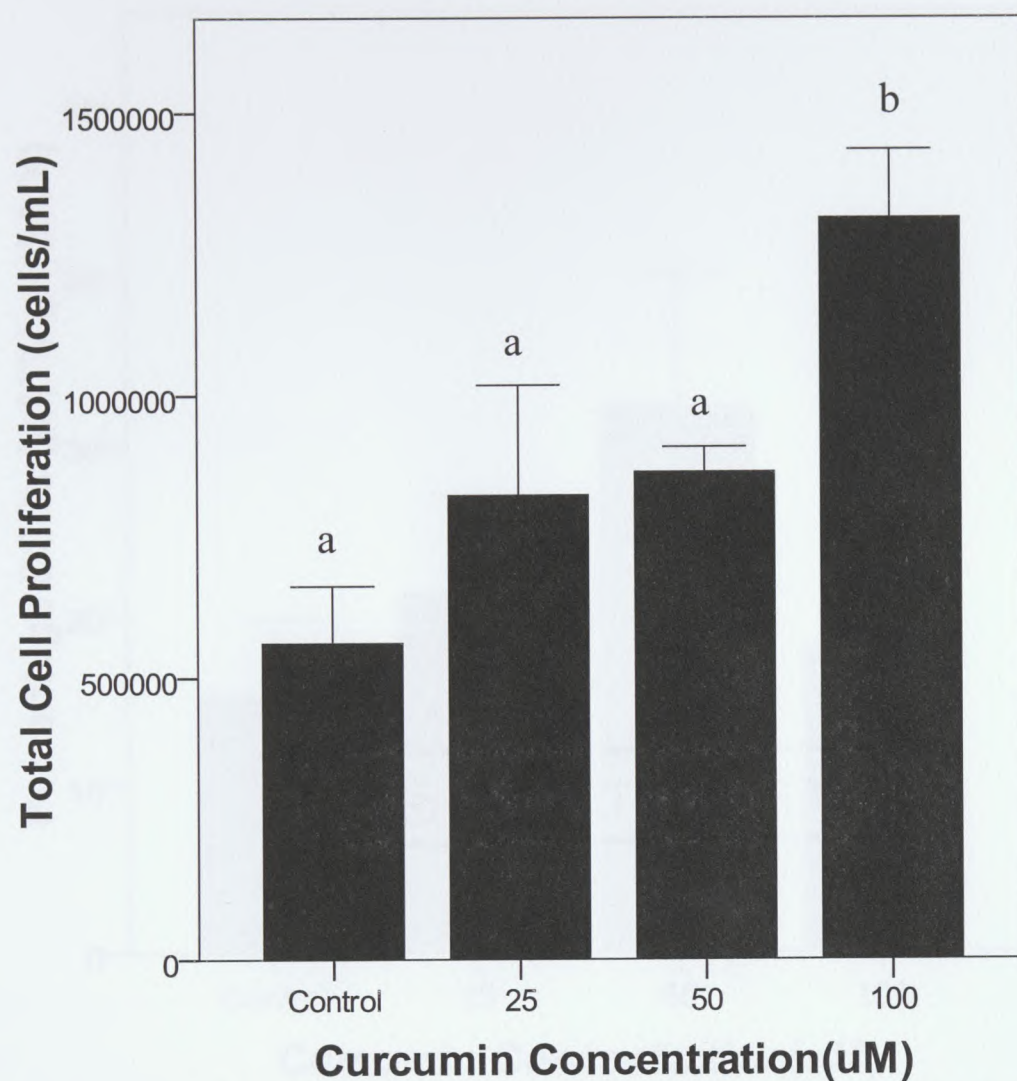


Figure 8. Effect of curcumin on average (± 1 S.E) proliferation of MDA-MB 468 cells. The x-axis corresponds to the different concentrations of curcumin measured in μM (0, 25, 50, and 100) and the y-axis corresponds to the total cell proliferation in cells/mL. Cells were treated with the above curcumin concentrations for 24 h. Proliferation was determined using the Annexin V Dead Cell kit and a Muse Cell Analyzer.

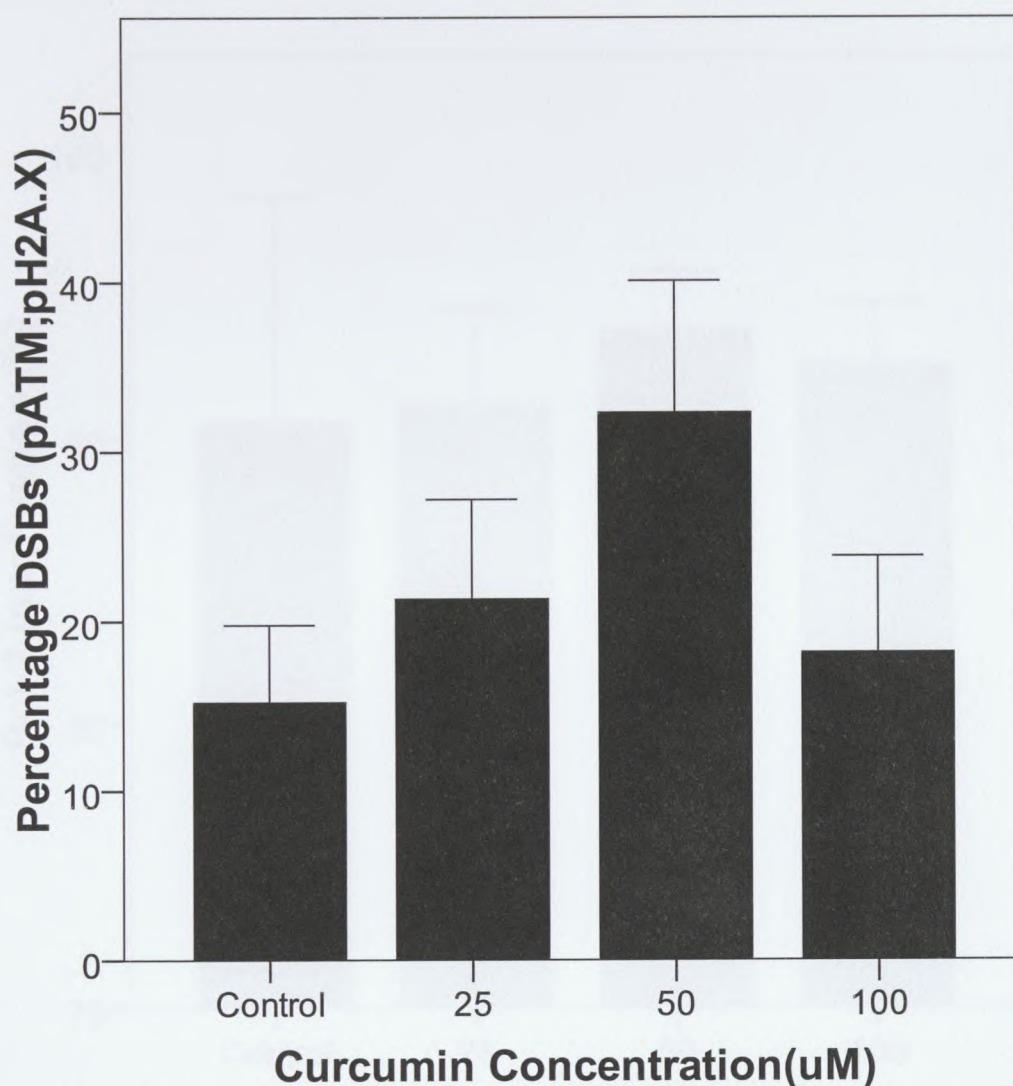


Figure 9. Effect of curcumin on average (\pm 1 S.E) p53 expression in MDA-MB 468 cells. Cells were exposed to 0, 25, 50, and 100 μ M curcumin for 24 hours to induce DNA damage, and then stained with both anti-phospho-Histone H2A.X and anti-phospho-ATM antibodies in multiplex. Indirect p53 expression was determined via percentage of double-strand breaks in DNA as a result of phosphorylation of the ATM and H2A.X proteins as seen on the y-axis. Samples were acquired using the MuseTM Cell Analyzer.

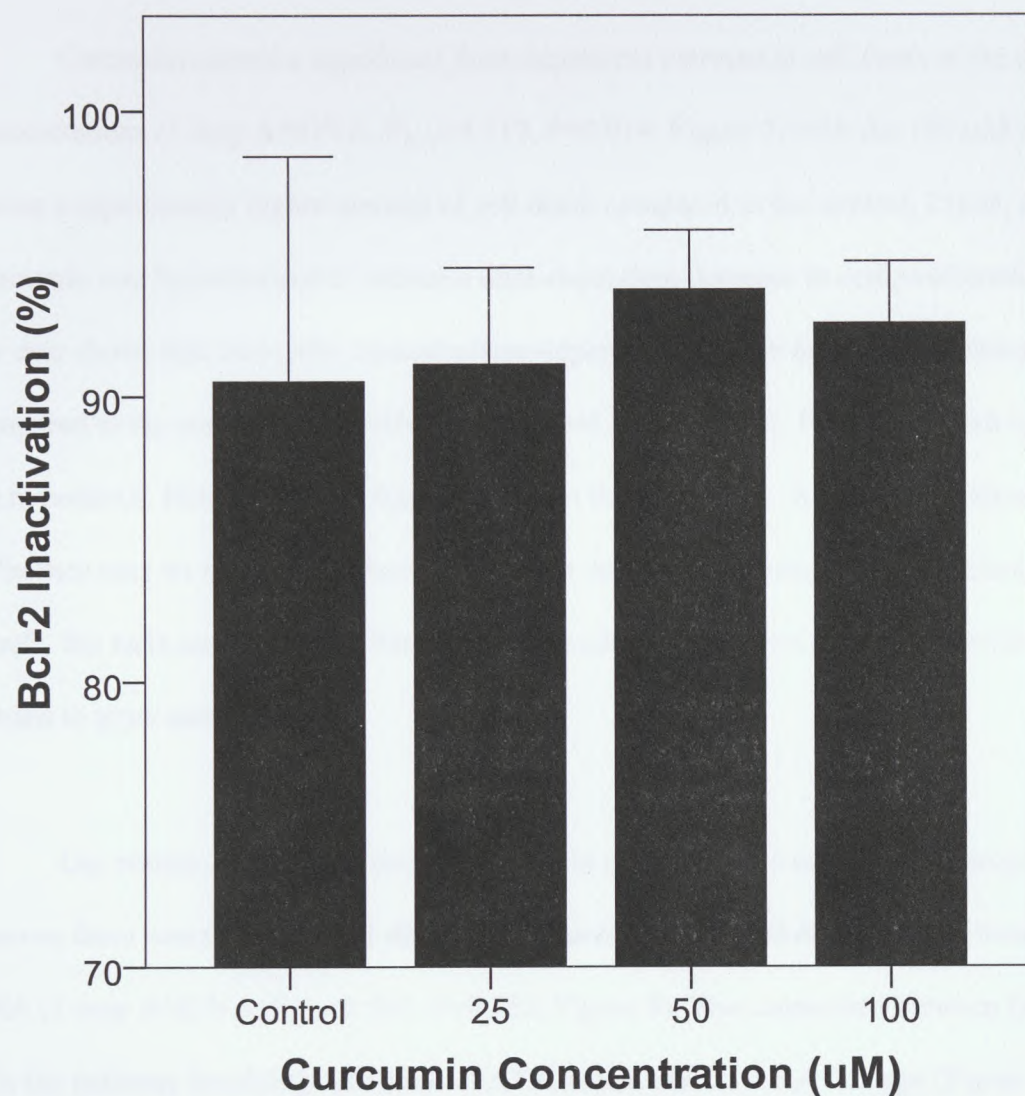


Figure 10. Effect of curcumin on average (\pm 1 S.E) expression of inactive Bcl-2 in MDA-MB 468 cells. Cells were exposed to 0, 25, 50, and 100 μ M curcumin for 24 hours to induce DNA damage, and then stained with both a phospho-specific anti-phospho-Bcl-2 and anti-Bcl-2 antibodies in multiplex. Samples were acquired using the MuseTM Cell Analyzer.

Discussion

Curcumin caused a significant dose-dependent increase in cell death at the different concentrations (1-way ANOVA, $F_{3,32}=4.119$, $P=0.014$; Figure 7) with the 100 μM concentration having a significantly higher amount of cell death compared to the control, 25 μM , and 50 μM . Curcumin was hypothesized to induce a dose-dependent decrease in cell proliferation. However, our data shows that curcumin caused a dose-dependent increase in cell proliferation when compared to the control (1-way ANOVA, $F_{3,32}=6.005$, $P=0.002$; Figure 8) which is contrary to the hypothesis. Hence, our data does not support the hypothesis. A possible explanation for this difference may be as follows. Since curcumin is derived from turmeric, a root plant containing starch, the cells could be using this starch as an additional carbon source to provide the energy needed to grow and reproduce.

Our results also suggest that curcumin did not cause an increase in p53 expression because there was no significant difference in curcumin-induced double strand breaks (DSB) in DNA (1-way ANOVA, $F_{3,32}=1.505$, $P=0.232$; Figure 9). The connection between DSB and p53 is in the pathway involving activation of ATM which detects DNA damage (Figure 1). When DNA damage occurs (curcumin-induced DSB) ATM phosphorylates the H2A.X protein which responds to DSBs. H2A.X then activates p53 to induce apoptosis or DNA repair. Therefore, an increase in DSBs should indirectly correspond to an increase in p53 expression. Furthermore, this increase in p53 expression should allow for p53 to initiate apoptosis because of a dose-dependent increase in DNA damage. Although a dose-dependent increase in DSB was shown in both the 25 μM and 50 μM curcumin treated samples relative to the control, the data was not statistically significant, $p=0.232$. Therefore, more trials need to be conducted in order to get a

significant result and prove the proposed pathway. In a study conducted by Rowe et al. in 2009, they found that following curcumin treatment, phosphorylation occurred at ATM-specific sites on the breast cancer (BRCA) 1 gene, which was consistent with the activation of a DNA damage response. In addition, phosphorylated H2A.X was detected in cells treated with curcumin, indicating that the type of damage induced by curcumin was a DNA double strand break (Rowe et al., 2009).

Curcumin did not cause a significant dose-dependent increase in inactive Bcl-2 expression as previously hypothesized (1-way ANOVA, $F_{3,20}=0.102$, $P=0.958$; Figure 10). It is known that curcumin induces apoptosis via suppression of anti-apoptotic Bcl-2, allowing the release of cytochrome c into the cytosol and activation of caspases (Anto et al., 2002; Rao et al., 2011). Inactivation (phosphorylation) of Bcl-2 allows the Bax protein to form a complex within the mitochondrial membrane that induces cell death. Although there was a slight increase in phosphorylated Bcl-2, it was not significant, $p=0.958$. Hence, more trials need to be conducted in order to see a more accurate reflection of the pathway taken by these cells to reach apoptosis.

Although there was no significant increase in p53 expression or Bcl-2 inactivation, there was a significant increase in both cell death and proliferation. Hence, future studies can look at the rates of curcumin-induced cell death versus cell growth in the cells. Also, the cells can be treated with curcumin for longer periods of time (24, 48, and 72 hours) which may result in curcumin inducing a higher percentage of DNA damage thereby decreasing the amount of cell growth. Also, curcumin's effects on p53 and Bcl-2 proteins should be studied on other carcinomas since these two oncoproteins play vital roles in cellular pathways.

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